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Thermal dependence of bioengineered glufosinate tolerance in cotton

James R. Mahan
Corresponding author. Plant Stress and Water Conservation Laboratory, USDA-ARS, 3810 Fourth Street, Lubbock, TX 79415-3397; jmahan@lbk.ars.usda.gov

Peter A. Dotray
Department of Plant and Soil Science, Texas Tech University, Box 42122, Lubbock, TX 79409-2122

Ginger G. Light
RR 1, Box 109, Petersburg, TX 79250

Kristy R. Dawson
Honors College, 103 McClellan Hall, Box 41017, Texas Tech University, Lubbock, TX 79409-1017

Tolerance to glufosinate has been bioengineered into cotton through the expression of a gene encoding the enzyme phosphinothricin acetyl transferase (PAT). Studies were conducted to determine thermal limitations on herbicide efficacy in bioengineered cotton. The 50% inhibition (I_{50}) of glufosinate of the target-site enzyme glutamine synthetase was thermally dependent with the lowest values between 25 and 35 C. Larger values of I_{50} were measured above and below the 25 to 35 C range. The apparent Michaelis constant K_M of the enzyme PAT was relatively stable from 15 to 30 C and increased more rapidly from 30 to 45 C. The two components in combination suggest the aggregate tolerance to glufosinate would not be thermally limited between 15 and 45 C. The thermal dependence of the aggregate tolerance in cotton suggests that glufosinate would not damage the crop over a range of temperatures. This prediction is in agreement with the results of field studies carried out over a number of years, which showed the glufosinate-tolerant cotton to be undamaged by glufosinate over a wide range of temperatures.

Nomenclature: Glufosinate; cotton, *Gossypium hirsutum* L. 'SeedCo 9023', 'DeltaPine 458'.

Key words: Glutamine synthetase, Michaelis-Menten constant, phosphinothricin acetyl transferase.

Control of weeds by herbicides is an integral part of modern agriculture (Monaco et al. 2002) and accounts for a significant input cost in many cropping systems. The high costs associated with weed control by herbicides are justified by the prevention of detrimental effects of competition between the crop and weed for water, sunlight, and nutrients. In light of the costs associated with weed control, it is critical that herbicide efficacy be consistent and effective regardless of the environmental conditions at the time of application. The control of weeds that are intermingled with crops is a critical component of control strategies, and this requirement to eliminate weeds growing in the presence of crop plants significantly complicates herbicidal weed control. The ability to apply an herbicide to both the weed and the crop is advantageous in many cropping systems and the use of selective herbicides is a common practice.

The development of herbicide-tolerant crops provides producers with additional options for weed control in crops. Commercially, herbicide-tolerant crops have been developed by two procedures: tolerance selection and bioengineering. Tolerance selection involves the identification of naturally occurring herbicide tolerance and the use of traditional breeding techniques to incorporate that tolerance into crops. The bioengineering of herbicide tolerance has been accomplished through the transfer of genes for the tolerance from one organism to another. Herbicide-tolerant crops that are a result of tolerance selection include sethoxydim- and imidazolinone-resistant corn (*Zea mays* L.) (Bernasconi et al. 1995; Dotray et al. 1993), which were developed to allow POST applications of sethoxydim or several imidazolinone herbicides. Examples of bioengineered herbicide-tolerant crops are the glufosinate- (Blair-Kerth et al. 2001) and glyphosate- (Bradshaw et al. 1997) tolerant technologies.

There are a variety of environmental factors that can reduce herbicide activity, and the result of diminished activity is evidenced by tolerance of the weed to the herbicide. Light et al. (1999) proposed that the thermal dependence of the action of herbicides could limit their efficacy in some environments and used the thermal dependence of the inhibition of a target enzyme by the herbicide to explain and predict tolerance under specific conditions. The thermal dependence of enzyme function characterized with respect to reaction rates and kinetic constants has been used to define thermal optima in plants. This information has been used to reduce the adverse effects of thermal variation on plants in terms of irrigation scheduling (Mahan et al. 2000), seedling emergence (Mahan 1994, 2000), and herbicide applications (Light et al. 1999, 2001). Knowledge of the effect of the thermal dependence of such herbicide tolerance was used to identify temperatures that would result in optimal weed control (Light et al. 2001). Mahan et al. (2004) proposed that similar temperature limits on enzyme function might adversely affect bioengineered herbicide tolerance.

Tolerance to glufosinate has been bioengineered into cotton (*Gossypium hirsutum* L.) (Blair-Kerth et al. 2001) through the expression of a gene encoding the enzyme phosphinothricin acetyl transferase (PAT). The introduced PAT metabolizes glufosinate into a compound without herbicide activity. In cotton that has been bioengineered to be tolerant to glufosinate, it is proposed that two mechanisms contribute to decreased glufosinate efficacy, an inherent kinetic tolerance (Mahan et al. 2004) and the bioengineered tolerance introduced by transgene expression (Blair-Kerth et al. 2001). Kinetic tolerance can be characterized in terms of the ther-

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

mal dependence of the inhibition of the target enzyme, glutamine synthetase (GS), by glufosinate defined by the thermal dependence of the I_{50} value (glufosinate concentration required to inhibit GS by 50%) for the inhibition as previously described for the pyrithiobac inhibition of acetolactate synthase by Light et al. (1999). The bioengineered tolerance will be characterized in terms of the thermal dependence of the apparent Michaelis constant (K_M) of PAT for the substrate glufosinate. This approach allows the identification of the temperature range over which herbicide efficacy is thermally limited and the plant would be predicted to be tolerant to glufosinate.

The objective of this study was to apply a similar analysis to thermal limitations on herbicide efficacy in a crop plant with a bioengineered herbicide tolerance in which a high level of tolerance is the desired outcome. The hypothesis in this study was that the engineered glufosinate tolerance in cotton is thermally dependent and that the thermal dependence could result in diminished herbicide tolerance under some thermal conditions.

Materials and Methods

Reagents

Glufosinate (Pestanal® analytical standard) was purchased from Riedel-de Haën.[1] Ignite® herbicide (18% active ingredient) was provided by BayerCropScience.[2] All other chemicals were reagent grade.

Plant Material

Cotton ('SeedCo 9023', which was bioengineered for glufosinate tolerance) was grown in a greenhouse before leaf harvest for PAT enzyme extractions. The fourth leaf below the terminal bud was used for extraction and assays. The absence of PAT activity in glufosinate susceptible cotton was verified (data not shown). GS was obtained from the fourth leaf below the terminal bud of cotton ('DeltaPine 458', which was glufosinate susceptible) grown in the greenhouse or in the field. Field-grown cotton generally had higher levels of extractable GS activity. Whole-plant efficacy was determined in glufosinate-susceptible cotton (DeltaPine 458).

PAT Extraction and Assay

The PAT extraction was modified for cotton from the method of De Block et al. (1987). All extractions were performed at 4 C. Plant tissue was ground at a ratio of 1 g of leaf to 4 ml of extraction buffer (0.025 M Tris-HCl pH 7.5, 0.001 M ethylenediaminetetraacetic acid (EDTA), 0.00008 M Leupeptin, 0.002 M phenylmethylsulfonylfluoride (PMSF), 0.00075 g/ml bovine serum albumin, 5% (w/v) polyvinylpolypyrolidone (PVPP), filtered through four layers of cheesecloth and centrifuged at 15,000 × *g* for 45 min at 4 C. The supernatant was precipitated with solid ammonium sulfate to 40% saturation (added over 1 h at 4 C) and centrifuged at 15,000 × *g* for 45 min. The resultant supernatant was precipitated with solid ammonium sulfate to 70% saturation (added over 1 h at 4 C) and centrifuged at 15,000 × *g* for 45 min. The 70% pellet was suspended in the extraction buffer and stored at −20 C before use in assays. The enzyme was stable for up to 1 wk under these conditions.

The PAT activity was assayed according to the method of Shaw (1975). The 1-ml assay mixture contained the following; 0.01 M Tris-HCl pH 7.8, 0.002 M 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), 0.0001 M acetyl-coenzyme A, 0.0002 M glufosinate ammonium, and 20 μl of the enzyme extract. Enzyme activity was monitored by the decrease in absorbance at 412 nm for 60 s, and the initial rate determined. Correction for deacylase activity used an assay mixture without glufosinate. A unit of activity was defined as 1 μmol of glufosinate acetylated min^{-1} at 25 C. Each assay was replicated a minimum of three times.

Thermal Dependence of the Apparent K_M of PAT

Thermal dependence of the K_M of glufosinate for PAT was determined at 5 C intervals over the temperature range from 15 to 45 C using concentrations of glufosinate ammonium from 12×10^{-6} to 5×10^{-4} M (encompassing the range from 0.5 K_M to 10 K_M). A minimum of three assays were performed for each inhibitor concentration at each temperature. The amount of PAT added to the assay mixture was adjusted at different temperatures to produce similar values of maximum velocity (V_{max}). The determination of the thermal dependence of the apparent K_M was repeated two times with PAT from two plant extractions. The value of the apparent K_M was determined from plots of initial velocity as a function of glufosinate ammonium concentration by computer-aided fitting to the Michaelis-Menten equation using Kalediagraph graphing/analysis software.[3]

GS Extraction and Assay

Leaves of glufosinate-susceptible cotton (100 g) were blended with 300-ml extraction buffer (0.1 M Tris-acetate 7.5 pH, 0.5 ml triton X-100, 0.005 M sodium glutamate, 0.01 M magnesium sulfate [$MgSO_4$], 0.001 M dithiothreitol [DTT], 0.001 M EDTA, and 1 g PVPP). The extract was filtered through four layers of cheesecloth and centrifuged at 15,000 × *g* for 45 min at 4 C. The supernatant was precipitated with solid ammonium sulfate to 20% saturation (added over 1 h at 4 C) and centrifuged at 15,000 × *g* for 45 min. The resultant supernatant was precipitated with solid ammonium sulfate to 50% saturation and centrifuged at 15,000 × *g* for 45 min. The 50% saturation pellet was resuspended in buffer (0.1 M Tris-acetate 7.5 pH, 0.005 M magnesium chloride [$MgCl_2$], 0.001 M DTT, and 0.001 M EDTA). The enzyme extract was desalted and concentrated by centrifugation through a Centricon Plus 20 (10 kd) centrifugal filter device[4] at 3,000 × *g* for 24 min at 4 C. The enzyme extract was stored (−20 C) before use, and activity was stable up to 2 wk under these conditions.

GS was measured with a coupled assay according to the procedure of Kingdon et al. (1968). The 1-ml assay mixture contained 0.05 M imidazole-HCl pH 7.1, 0.04 M NH_4Cl_2, 0.1 M sodium glutamate, 0.001 M phosphoenolpyruvate (PEP), 0.05 M $MgCl_2$, 0.01 M KCl, 0.00035 M NADH, 10 units pyruvate kinase, and 49 units lactate dehydrogenase. The reaction was initiated by the addition of 100 μl of 0.076 M ATP (0.0076 M assay concentration) and 20 μl of the GS extract. The progress of the reaction was monitored at 340 nm for 180 s. A unit of activity was defined as 1 μmol of glutamine consumed per min at 30 C.

Thermal Dependence of Glufosinate I_{50}

Inhibition of GS activity by glufosinate was determined at concentrations of glufosinate varying from 10^{-7} to 0.1 M. A minimum of three assays were carried out at each concentration. The I_{50} values were determined at 5 C intervals over the temperature range from 15 to 45 C. The I_{50} value is defined as the inhibitor concentration at which the activity is half the activity of the reaction without the inhibitor. I_{50} values were determined from a computer-generated curve fit of reaction rate and inhibitor concentration using Kalediagraph graphing/analysis software. The determination of the thermal dependence of the I_{50} was repeated two times with GS from two plant extractions.

Herbicide Treatment

Glufosinate-susceptible cotton was planted in soil in 4-L containers in a greenhouse and grown for 30 d until plants developed three leaves. The plants were watered twice each week with deionized water. Before herbicide treatment, plants were thinned to two plants per container.

At 8:00 A.M., 1 h before the herbicide treatment, the plants were transferred to growth chambers set to 18, 27, or 40 C. The 1-h equilibration period was used to allow the plant to come to a stable temperature before the herbicide application. Light intensity, provided by a mixture of incandescent[5] and fluorescent[6] lamps, was 150 μmol m^{-2} s^{-1} parabolic aluminized reflector at plant height. After the 1-h equilibration period the plants were sprayed with glufosinate at 88 g ai ha^{-1} with a backpack sprayer calibrated to deliver 140 L ha^{-1} at 129 kPa with 80015 nozzles. Immediately following herbicide application, the plants were returned to chambers set at 18, 27, or 40 C for an 8-h posttreatment period. After this period, the plants were returned to the greenhouse. The 1-h pretreatment equilibration was used in a previous study by Mahan et al. (2004), and the 8-h posttreatment period resulted from preliminary studies (data not shown) that indicated that 8 h resulted in maximal differences among application temperatures. Two pots with two plants per pot were used in each application, and the experiment was repeated two times.

Leaf Damage Assessment

Four days after herbicide treatment, cotton injury was quantified in terms of the percentage of the leaf area that was damaged by glufosinate. The leaves of the plants were individually photographed, and the fraction of the area of each leaf that was damaged was measured.[7] At this stage, leaf damage appeared as necrotic spots on the leaves.

Results and Discussion

Thermal Dependence of the K_M of PAT

There was no detectable PAT activity in the nontransgenic, glufosinate-susceptible cotton (data not shown). Figure 1 illustrates the effect of thermal variation on the apparent K_M of the enzyme PAT for the substrate glufosinate in glufosinate-tolerant cotton. The apparent K_M increased with temperature across the 15 to 45 C range. The K_M value was relatively stable from 15 to 30 C and increased more rapidly from 30 to 45 C. The K_M increases slightly more than twofold over the entire temperature range. Increases in the K_M of a substrate for an enzyme have been previously proposed to indicate a decline in enzyme function and a resultant decline in reaction rate. Mahan et al. (1990) suggested that a twofold increase in the apparent K_M value was indicative of a transition from optimal to nonoptimal enzyme function and an indicator of temperature stress. Because it is the activity of PAT that provides the tolerance to glufosinate, a decline in the reaction rate could indicate a limitation on the tolerance mechanism. Under this criterion it could be predicted that PAT-catalyzed glufosinate metabolism could begin to experience a thermal limitation at temperatures between 40 and 45 C. Because canopy temperatures in excess of 40 C rarely occur in cotton in the field, the PAT-based glufosinate tolerance would not be expected to be thermally limited, particularly for cotton in the absence of water stress.

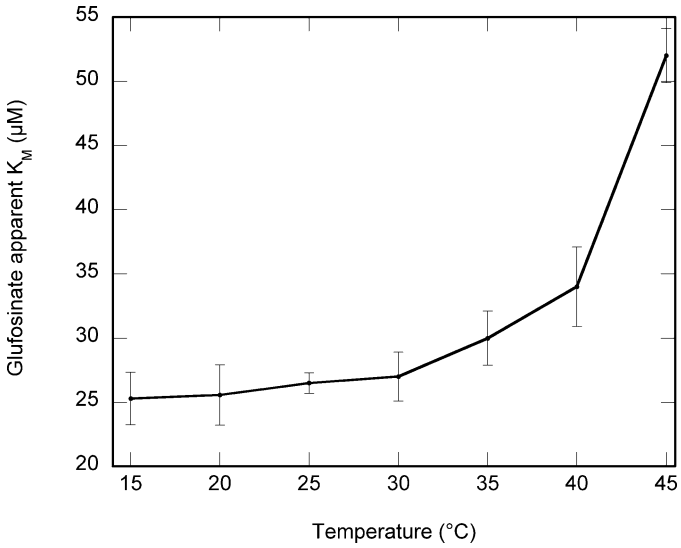

FIGURE 1. Thermal dependence of the apparent K_M (± SE) for glufosinate of the PAT from cotton with bioengineered glufosinate tolerance. The concentration of glufosinate was varied from 0.5 to 10 times K_M at each temperature.

Thermal Dependence of Glufosinate I_{50}

The inhibition of GS by glufosinate is shown in Figure 2. The I_{50} of the herbicide for the inhibition of GS was thermally dependent, with the lowest values between 25 and 35 C. Higher values of I_{50} were measured above and below the 25 to 35 C range. The observed pattern of I_{50} variation, a temperature range of minimal I_{50} values bracketed by higher values above and below that range, was similar to that noted for the I_{50} of pyrithiobac for acetolactate synthase (Light et al. 1999). These data suggest that inhibition of GS by glufosinate is optimal in the 25 to 35 C range and potentially subject to thermal limitations above and below that range. Canopy temperatures in the 25 to 35 C range are common for cotton in the field (Light et al. 2001). Temperatures below 25 C are common in many cotton growing regions, and low temperatures could be expected to reduce glufosinate efficacy and thus enhance the observed tolerance. Temperatures above 35 C are uncommon for cotton in the field within semiarid environments.

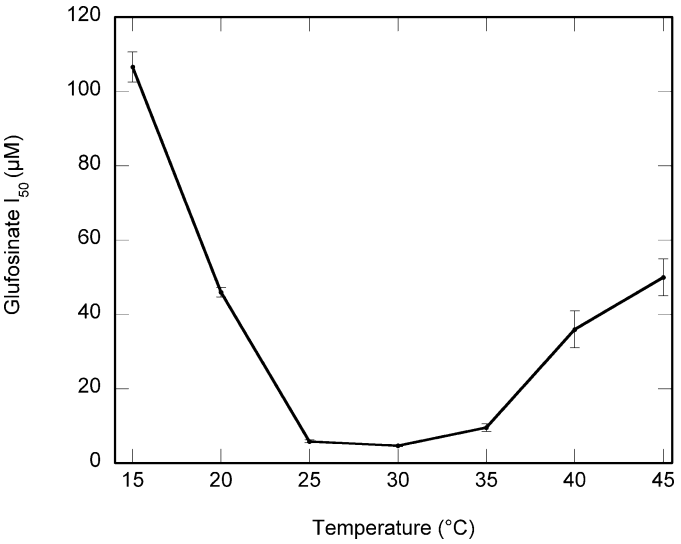

FIGURE 2. Inhibition of glutamine synthetase by glufosinate in glufosinate-susceptible cotton. The glufosinate concentration varied from 10^{-7} to 0.1 M.

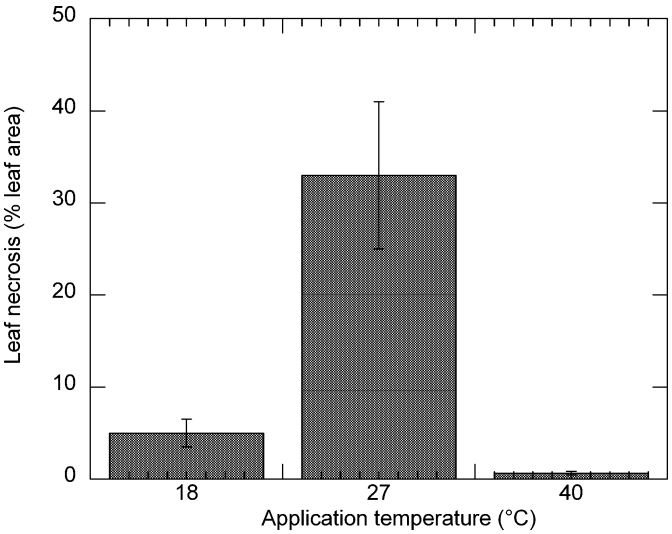

FIGURE 3. Temperature dependence of glufosinate efficacy (leaf necrosis ± SE) in glufosinate-susceptible cotton. Plants were grown in a greenhouse for 30 d before glufosinate treatment. Plants were equilibrated to the application temperature for 1 h before treatment and remained at the treatment temperature for 8 h following application then were returned to greenhouse for 4 d before quantification of leaf necrosis.

Kinetic Tolerance to Glufosinate in Cotton

In whole plant studies, the efficacy of glufosinate in glufosinate-susceptible cotton was highest at 27 C and reduced at 18 and 40 C (Figure 3). Glufosinate did not damage the leaves of the glufosinate-tolerant cotton at any temperature (data not shown). We propose that the low efficacy in the glufosinate susceptible may, in part, be attributed to the kinetic tolerance resulting from elevated I_{50} values whereas in the glufosinate-tolerant cotton the low efficacy is an indication of the "aggregate tolerance" resulting from the combination of the kinetic tolerance and the PAT-derived tolerance. A similar study in Palmer amaranth (*Amaranthus palmeri* S. Watts) yielded a similar correlation between the thermal dependence of the I_{50} and that of efficacy (Light et al. 1999, 2001). The combined results indicate that the efficacy of glufosinate on cotton is limited at temperatures that are coincident with increasing I_{50} values determined in the laboratory. The kinetic tolerance was minimal in the 25 to 35 C range and greatest at temperatures outside this range.

In cropping systems that include a herbicide-tolerant crop, the herbicide will be applied to both the weeds and the crop; each of which has the potential to be adversely affected. Although a great deal of effort has been expended by the developers of herbicide-tolerant crops to ensure that they are unaffected by the herbicide, in practice, there are limitations on the tolerance, and herbicide damage may be observed.

In previous studies, Light et al. (1999, 2001) reported that the thermal dependence of the inhibition of a target enzyme by a herbicide can reduce efficacy and that the resultant tolerance could result in a reduced ability to control weeds. This kinetic tolerance could be expected to be a characteristic of a plant with a herbicide that relies on enzyme inhibition for its mode of action. The agronomic result of this kinetic tolerance depends on whether it is observed in a weed or in a crop plant. In a weed, an inability of the herbicide to damage the plant is an undesirable outcome, whereas the same kinetic tolerance in a crop plant is a highly desirable outcome (i.e., the herbicide will not damage the plant). In the cotton with bioengineered glufosinate tolerance used in this study, there are two enzyme-based mechanisms that contribute to the observed herbicide tolerance: the well-documented effect of the introduced PAT activity that metabolizes glufosinate, and the previously proposed kinetic tolerance resulting from limitations on the inhibition of target enzyme inhibition by glufosinate (Mahan et al. 2004). Given that both mechanisms involve enzymes, they are potentially temperature dependent, and it is possible that the herbicide tolerance could be adversely affected under some thermal conditions. The characterization of the thermal dependence of glufosinate tolerance in bioengineered cotton reported in this article involved the characterization of two thermal dependencies: (1) the glufosinate tolerance due to the effect of temperature on the metabolism of glufosinate by PAT, and (2) the kinetic tolerance due to the effect of temperature on the ability of glufosinate to inhibit the GS, the target enzyme for glufosinate.

Regardless of the underlying mechanism, the ability of a plant to survive a herbicide application was observed as a phenotype with low-herbicide efficacy. Without knowledge of the specific mechanism that produces the reduced herbicide efficacy, the low-efficacy phenotype that was observed cannot be fully defined. In a crop with bioengineered tolerance to an herbicide, the bioengineered tolerance and kinetic tolerance combine to produce an "aggregate tolerance" that results in the observed phenotype. Ultimately, the success of the bioengineered herbicide-tolerant plant subjected to applications of herbicide within a variable thermal environment will be determined by the range of temperatures over which the aggregate tolerance provides sufficient protection against the herbicide. The thermal dependence of the aggregate tolerance for the glufosinate-tolerant cotton in this study is shown in Figure 4. The PAT-related tolerance was present over the range from 15 to 40 C, and the kinetic tolerance is possible at temperatures above and below the 25 to 35 C range. The two components in combination suggest the aggregate tolerance to glufosinate would not be

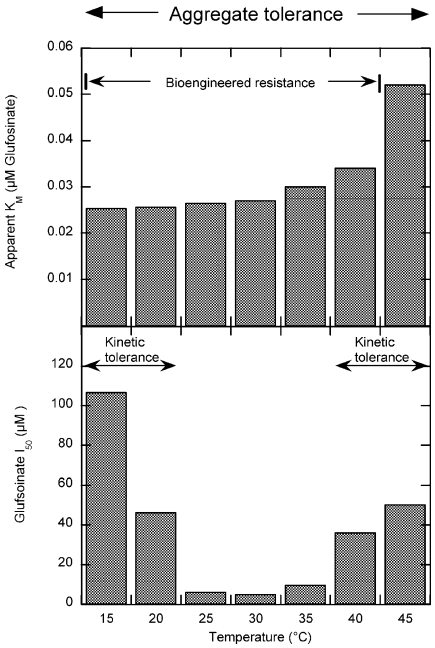

FIGURE 4. Aggregate tolerance to glufosinate in glufosinate-tolerant cotton. Aggregate tolerance indicates the combined effect of thermal variation in apparent Michaelis constant K_M of phosphinothricin acetyl transferase (PAT) and the 50% inhibition (I_{50}) of glufosinate for glutamine synthetase (GS).

thermally limited between 15 and 45 C, the entire thermal range investigated in this study.

The thermal dependence of the aggregate tolerance in cotton suggests that glufosinate would not damage the crop over a range of temperatures that could be expected to occur for cotton in the semiarid growing region of the Southern High Plains of Texas. This prediction is in agreement with the results of field studies carried out over a number of years, which showed the glufosinate-tolerant cotton to be undamaged by glufosinate applications over a wide range of temperatures (Blair-Kerth et al. 2001). Given that the thermal dependence of herbicide tolerance can be determined by field studies, what are the advantages of using a kinetic analysis to define the thermal dependence? Perhaps the most obvious advantage of a kinetic analysis approach is that it can be accomplished in a period of weeks as opposed to the months of analysis over successive years that are required for a field study. The ability to carry out kinetic studies during the development of the herbicide-tolerant crop could perhaps be useful in assessing the suitability of approaches to tolerance in the early stages of development.

Sources of Materials

[1] Glufosinate (Pestanal® analytical standard), Riedel-de Haën, Wunstorfer Straze 40, D-30926 Seelze, Germany.

[2] Glufosinate (Ignite®), Bayer CropScience LP, P.O. Box 12014, 2 T.W. Alexander Drive, Research Triangle Park, NC 27709.

[3] Kalediagraph graphing/analysis software, Synergy Software, 2457 Perkiomen Avenue, Reading, PA 19606.

[4] Centricon Plus 20 (10 kd) centrifugal filter device, Millipore Corporation, 290 Concord Road, Billerica, MA 01821.

[5] 10 Sylvania Softwhite 60 W incandescent bulbs, 100 Endicott Street, Danvers, MA 01923.

[6] 16 GE F72T12-CW 1500 1.82-m fluorescent lamp bulbs, General Electric Company, 3135 Easton Turnpike, Fairfield, CT 06828-0001.

[7] NIH Image software, Research Services Branch, National Institute of Mental Health, National Institutes of Health, 6001 Executive Boulevard, Room 8184, MSC 9663 Bethesda, MD 20892-9663. http://rsb.info.nih.gov/nih-image.

Acknowledgments

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